MicroRNA-31 Promotes Skin Wound Healing by Enhancing Keratinocyte Proliferation and Migration

Dongqing Li[1], Xi Li[1], Aoxue Wang[1,2], Florian Meisgen[1], Andor Pivarcsi[1], Enikö Sonkoly[1], Mona Ståhle[1] and Ning Xu Landén[1]

Wound healing is a basic biological process restoring the integrity of the skin. The role of microRNAs during this process remains largely unexplored. By using an *in vivo* human skin wound healing model, we show here that the expression of miR-31 is gradually upregulated in wound edge keratinocytes in the inflammatory (1 day after injury) through the proliferative phase (7 days after injury) in comparison with intact skin. In human primary keratinocytes, overexpression of miR-31 promoted cell proliferation and migration, whereas inhibition of miR-31 had the opposite effects. Moreover, we identified epithelial membrane protein 1 (EMP-1) as a direct target of miR-31 in keratinocytes. The expression of EMP-1 in the skin was negatively correlated with the level of miR-31 during wound healing. Silencing of EMP-1 mimicked the effects of overexpression of miR-31 on keratinocyte proliferation and migration, indicating that EMP-1 is a critical target mediating the functions of miR-31 in keratinocytes. Finally, we demonstrated that transforming growth factor-β2, which is highly expressed in skin wounds, upregulated miR-31 expression in keratinocytes. Collectively, we identify miR-31 as a key regulator for promoting keratinocyte proliferation and migration during wound healing.

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INTRODUCTION

Cutaneous wound healing is a highly orchestrated biological process, which is essential to restore the integrity of the skin barrier. The classical model of wound healing consists of four sequential, yet overlapping phases: hemostasis (up to several hours after injury), inflammation (1–3 days after injury), proliferation (4–21 days after injury), and tissue remodeling (21 days-1 year after injury; Gosain and DiPietro, 2004). Re-epithelialization is a crucial process, which entails resurfacing of the wound with new epithelium and requires proper migration and proliferation of keratinocytes at the periphery of the wound (Santoro and Gaudino, 2005). Defects of re-epithelialization are associated with chronic non-healing wounds, which affect about 1–2% of the population in developed countries and present a major and rising health and economic burden to society (Sen *et al.*, 2009). The complex nature of wound healing process makes it challenging to develop effective therapies. Current treatments for chronic ulcers focus mainly on optimization of controllable healing factors—e.g., nutritional support, infection clearance, and mechanical support. Few targeted approaches have been developed—e.g., topical application of growth factor, such as platelet-derived growth factor (Sun *et al.*, 2014a).

MicroRNAs (miRNAs) are ~22nt noncoding RNAs, which are being recognized as important gene regulators during the last decade (He and Hannon, 2004). In humans, miRNAs are proposed to regulate ~60% of all protein-coding genes (Friedman *et al.*, 2009) and are thus involved in most biological processes investigated—e.g., development, organogenesis, apoptosis, and cell proliferation (Iorio and Croce, 2012; Ha and Kim, 2014). Deregulation of miRNA expression has been implicated in the pathogenesis of many diseases, such as cancer, infection, and chronic inflammatory diseases (Bertero *et al.*, 2011; Iorio and Croce, 2012; O'Connell *et al.*, 2012). Increasing pre-clinical and clinical studies have demonstrated that modulation of miRNA expression by administration of specific miRNA mimics or inhibitors may be beneficial for treating diseases (Hydbring and Badalian-Very, 2013; Nana-Sinkam and Croce, 2013). Thus, miRNAs represent potentially important therapeutic targets.

Although in recent years increasing studies have revealed the important roles of miRNAs in skin biology and diseases (Schneider, 2012), the understanding of their role in the wound healing process is still in its infancy. MiR-31 has been implicated as an important regulator of keratinocyte

[1]*Molecular Dermatology Research Group, Unit of Dermatology and Venereology, Centre for Molecular Medicine (CMM), Department of Medicine, Karolinska Institutet, Stockholm, Sweden and* [2]*Department of Dermatology, The Second Affiliated Hospital of Dalian Medical University, Dalian, China*

Correspondence: Ning Xu Landén, Molecular Dermatology Research Group, Unit of Dermatology and Venereology, Centre for Molecular Medicine (CMM), L8:02, Department of Medicine, Karolinska Institutet, Stockholm SE-17176, Sweden. E-mail: ning.xu@ki.se

Abbreviations: miRNA, microRNA; EMP-1, epithelial membrane protein 1; pri-mir-31, primary miR-31 transcript; qRT-PCR, real-time quantitative reverse transcription–PCR; siRNA, small interfering RNA; TGF-β, transforming growth factor-β

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biology—e.g., differentiation and hair growth (Mardaryev *et al.*, 2010; Peng *et al.*, 2012). Moreover, the upregulation of miR-31 has been reported in several skin diseases characterized by excessive keratinocyte proliferation—e.g., psoriasis, a chronic inflammatory skin disease sharing many features with wound healing (Xu *et al.*, 2012; Morhenn *et al.*, 2013) and cutaneous squamous cell carcinoma (Bruegger *et al.*, 2013; Wang *et al.*, 2014). We hypothesize that miR-31 may also play a role during skin wound healing—e.g., by regulating keratinocyte proliferation. Therefore, here we focused on miR-31 and studied its expression and function in human skin wound healing. We show that miR-31 is gradually upregulated in wound edge keratinocytes in the inflammatory phase and proliferative phase of wound healing and that it promotes cell proliferation and migration by suppressing its direct target gene, epithelial membrane protein 1 (EMP-1). We identify transforming growth factor-β2 (TGF-β2), which is highly expressed in the wound bed, as an inducer of miR-31 expression. Taken together, our study suggests that miR-31 may be a potential therapeutic target for promoting skin wound healing.

RESULTS

MiR-31 is upregulated in epidermal keratinocytes during human skin wound healing

To study the expression of miRNAs during human skin wound healing, we created surgical wounds in the abdominal skin of 17 healthy volunteers (Supplementary Table S1 online). Wound edge tissues were collected 1 day (inflammatory phase) and 7 days (proliferative phase) after injury (Supplementary Figure S1 online). The histological changes of the human skin wound during healing process are shown in Supplementary Figure S2 online.

Herein, we focused on miR-31 and characterized its expression pattern throughout the entire healing process. Real-time quantitative reverse transcription–PCR (qRT-PCR) analysis revealed that the basal level of miR-31 in human skin is low but rapidly increasing 1.9-fold 1 day after injury ($P=0.008$; Figure 1a). In the subsequent proliferative phase (7 days post wounding), the expression of miR-31 was continuously upregulated 7.7-fold ($P=0.003$) compared with the unwounded skin. Moreover, in the same wound biopsies, we analyzed the expression of primary miR-31 transcripts (pri-miR-31), from which mature miR-31 is processed (Figure 1b). Pri-miR-31 was increased in the proliferative phase (5.6-fold, $P=0.007$) compared with the unwounded skin (Figure 1b), positively correlating with the level of mature miR-31 ($R=0.69$, $P=0.0017$; Figure 1c).

To identify the cell type(s) responsible for the dynamic changes of miR-31 expression during wound healing, we performed *in situ* hybridization (Figure 1d). In line with the qRT-PCR results, the expression of miR-31was low in unwounded skin (day 0), and its signal was mainly localized in the basal layer of epidermal keratinocytes. The level of miR-31 was markedly upregulated in the proliferative phase (day 7), spreading to the suprabasal cell layers at the wound edge. Minor amounts of miR-31 may also be present in dermal fibroblasts and infiltrating immune cells. To clarify the expression pattern in detail, we separated the epidermis and dermis of the wound biopsies from healthy donors ($n=5$) by using laser capture microdissection and analyzed the expression of miR-31 by qRT-PCR (Figure 1e and Supplementary Figure S3 online). Our results show that miR-31 expression is mainly upregulated in the epidermis compared with the dermis during wound healing, which is in line with the PCR data of miR-31 expression in whole skin biopsies (Figure 1a) and *in situ* hybridization (Figure 1d). Taken together, our results demonstrate that epidermal keratinocytes are the primary cells of interest for functional studies of miR-31 during skin wound healing.

MiR-31 promotes keratinocyte proliferation

Given that miR-31 is highly upregulated in keratinocytes at the wound edge in the proliferative phase, we further studied the role of miR-31 in keratinocyte proliferation. To this end, we overexpressed or inhibited miR-31 in human primary keratinocytes by transient transfection of miR-31 specific precursor RNA (Pre-miR-31) or inhibitor (Anti-miR-31), respectively. The efficiency of overexpression or inhibition of miR-31 was confirmed by qRT-PCR (Supplementary Figure S4 online).

We performed the CyQUANT cell proliferation assay for the keratinocytes with modified miR-31 expression, which showed that overexpression of miR-31 increased, whereas inhibition of miR-31 decreased the number of viable cells in culture (Figure 2a and Supplementary Figure S5 online). We also observed the increased expression of the proliferation marker Ki-67 in keratinocytes with overexpressed miR-31, whereas Ki-67 was reduced upon miR-31 inhibition (Figure 2b). These results were further confirmed by the 5-ethynyl-2′-deoxyuridine (EdU) incorporation assay. Inhibition of miR-31 decreased cell proliferation, as evidenced by the reduced percentage of EdU-positive cells and the cells within S-phase (Figure 2c). Furthermore, using colony formation assays, we found that miR-31 enhanced the long-term growth of keratinocytes (Figures 2d and e). Collectively, we demonstrate that miR-31 is a positive regulator of keratinocyte growth, reflected by both immediate increase of cell cycle progression and long-term promotion of self-renewal.

MiR-31 enhances keratinocyte motility

Migration of keratinocytes is one of the essential biological processes for epidermal repair (Haase *et al.*, 2003); therefore, we aimed to determine the role of miR-31 in keratinocyte migration. To that end, we performed scratch assays that showed that overexpression of miR-31 significantly accelerated the healing rate of keratinocytes 24 hours after scratching (1.84-fold, $P=0.0001$; Figure 3a), whereas inhibition of endogenous miR-31reduced the rate of keratinocyte migration (0.19-fold, $P=0.004$ at 7 hours; 0.33-fold, $P=0.00001$ at 24 hours; Figure 3b and Supplementary Figure S5 online). In line with this, by using the haptotactic transwell migration assay, we found that overexpression of miR-31strikingly increased (3.6-fold, $P=0.04$) the migration capacity of keratinocytes (Figure 3c). *Vice versa*, inhibition of miR-31 resulted in a significant decrease (0.33-fold, $P=0.003$) in cell

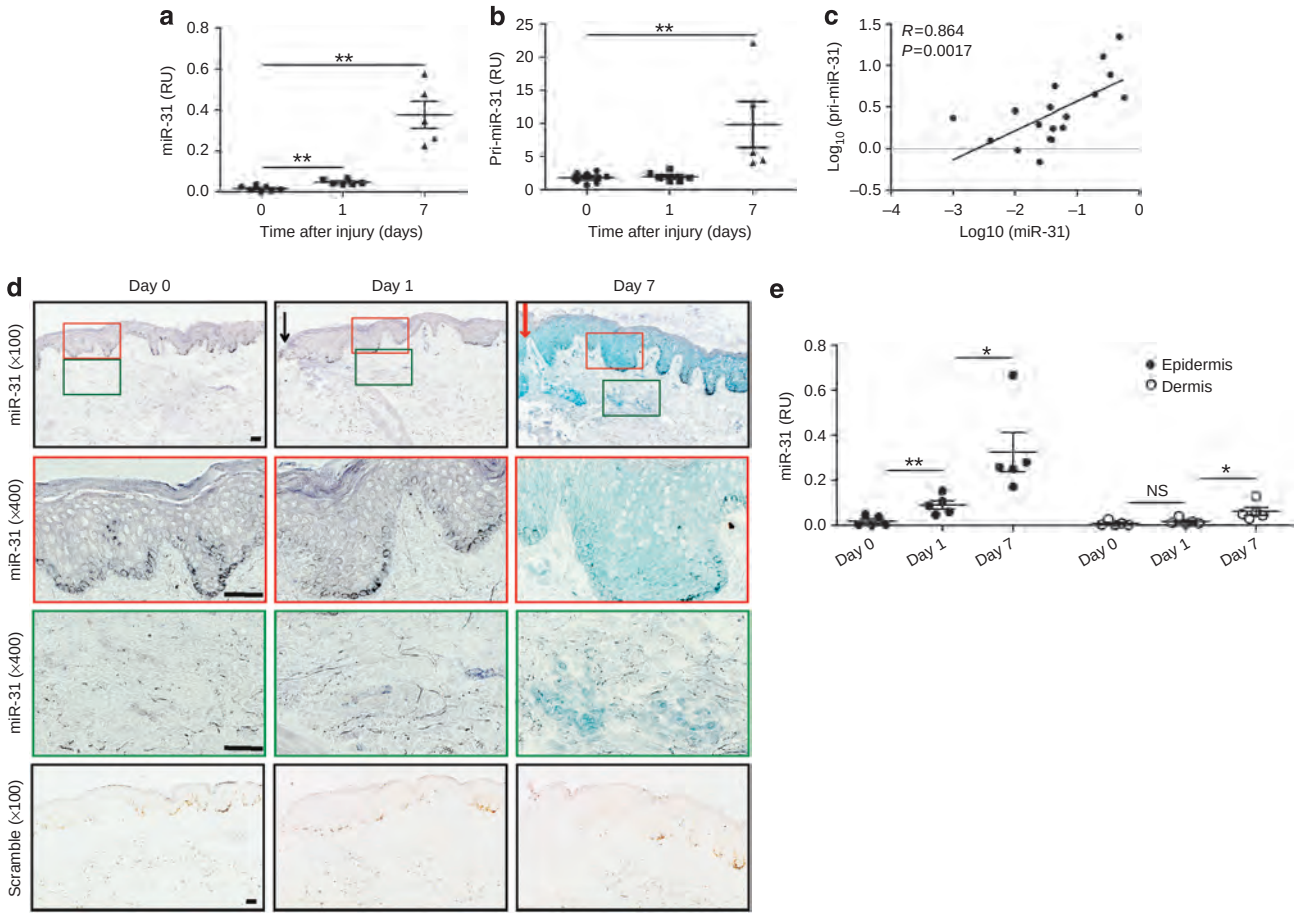

Figure 1. MiR-31 is upregulated during skin wound healing. The expressions of miR-31 (**a**) and of primary miR-31 transcript (pri-miR-31; **b**) were analyzed in wound biopsies from healthy donors ($n=7$) before or 1 and 7 days after injury using real-time quantitative reverse transcription–PCR (qRT-PCR). (**c**) Correlation of miR-31 with pri-miR-31 expression in human wound biopsies 1 and 7 days after injury, Spearman-correlation on log-transformed values. (**d**) *In situ* hybridization was performed on wound biopsies from healthy donors ($n=7$) using miR-31-specific probe or scrambled probe. Blue-green color indicates miR-31 expression. Black arrow demarcates wound edges on day 1, whereas red one points at newly formed epidermis on day 7 post wounding. Scale bar = 100 μm. (**e**) The Epidermis and dermis in wound biopsies from healthy donors ($n=5$) before or 1 and 7 days after injury were separated by laser capture microdissection. The expression of miR-31 were analyzed using qRT-PCR. MiR-31 and pri-miR-31 PCR data are expressed in relative units (RU) compared with RNU48 RNA and 18S ribosomal RNA, respectively. Results for individual donors and mean ± SD are shown. $^{*}P<0.05$ and $^{**}P<0.01$; the Mann–Whitney *U*-test.

migration (Figure 3d). Together, our data identify miR-31 as a positive regulator of keratinocyte motility.

EMP-1 is targeted by miR-31 in keratinocytes

MiRNAs exert biological functions through regulating their target genes. Thus, we aimed to identify the target gene mediating the biological effects of miR-31 in keratinocytes. To predict the potential targets for miR-31, we performed an integrated analysis using three independent algorithms: TargetScan (Lewis *et al.*, 2005), miRanda (John *et al.*, 2004), and miRWalk (Dweep *et al.*, 2011). Hundred putative miR-31 targets were commonly predicted by all three algorithms (Figure 4a and Supplementary Table S2 online). To identify targets that are regulated by miR-31 in keratinocytes, we revisited the data from our previous study (Xu *et al.*, 2012), in which transcriptome analysis was performed on keratinocytes after inhibition of miR-31 (GSE41905). Among the 100 targets commonly predicted by all three algorithms, four genes were significantly upregulated (fold change ⩾ 1.2, $P<0.05$) upon miR-31 inhibition (Supplementary Table S2 online). In this study, we focused on one of them, EMP-1, as it had been previously identified as a direct target of miR-31 in esophageal squamous cell carcinoma (Zhang *et al.*, 2011), containing a conserved binding site for miR-31 in its 3′-untranslated region (3′-UTR) of mRNA (Figure 4b; Lewis *et al.*, 2005). Notably, EMP-1 has been shown to regulate cancer cell proliferation and migration in several previous studies (Zhang *et al.*, 2011; Sun *et al.*, 2013; Sun *et al.*, 2014b; Sun *et al.*, 2014c; Sun *et al.*, 2014d).

First, we examined whether EMP-1 was directly targeted by miR-31 in human primary keratinocytes. We performed 3′-UTR luciferase reporter assays with reporter gene constructs containing the full-length 3′-UTR of EMP-1 mRNA in human primary keratinocytes (Figures 4b and c). The wild-type EMP-1 3′-UTR luciferase activity was decreased (0.78-fold, $P=0.009$) by miR-31 overexpression, whereas mutation

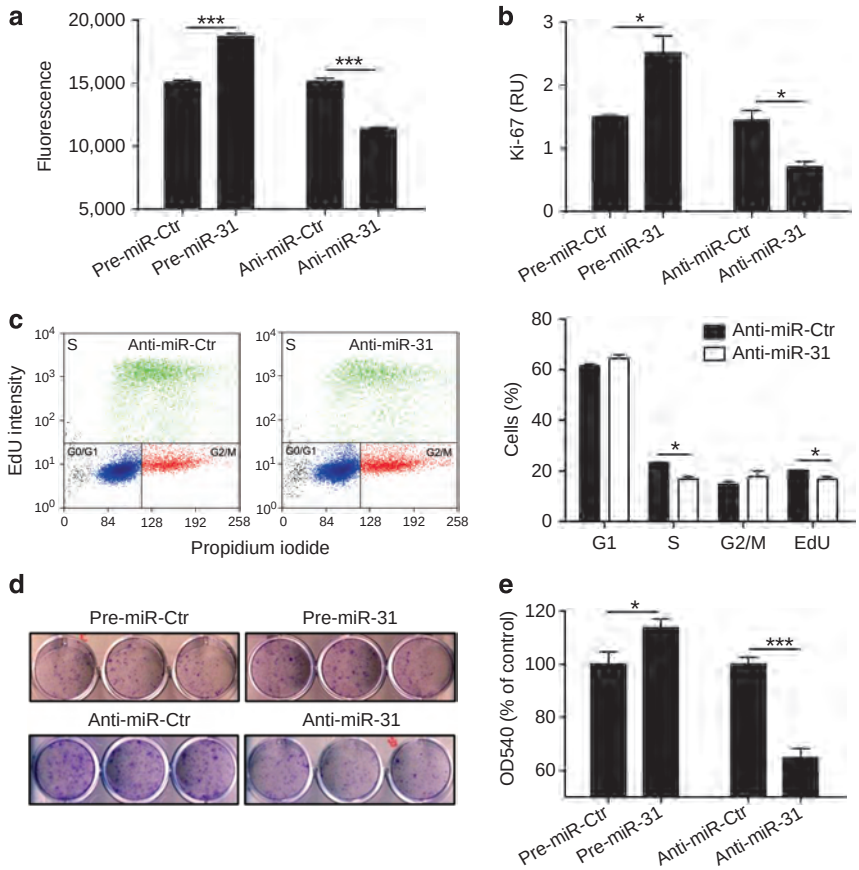

Figure 2. MiR-31 promotes keratinocyte proliferation. (**a**) Keratinocytes were transfected with 20 nM pre-miR-31/anti-miR-31 for 48 hours. The number of viable cells in culture was quantified by the CyQUANT cell proliferation assay. (**b**) The expression of proliferation marker Ki-67 was analyzed in the transfected keratinocytes using real-time quantitative reverse transcription–PCR. Data are expressed in relative units (RU) compared with 18 s ribosomal RNA with mean ± SD (**c**) Keratinocytes were transfected with 20 nM anti-miR-31 or anti-miR-control (Ctr) for 48 hours. Cell proliferation and cell cycle progression were measured by 5-ethynyl-2′-deoxyuridine (EdU) labeling and subsequent cell cycle analysis by flow cytometry. Percentage of cells in the G1, S, and G2/M phase of cell cycle and percentage of EdU+ cells are shown. (**d**) Colonies formed by the transfected keratinocytes were stained with crystal violet 8 days after transfection. (**e**) Crystal violet was dissolved and absorbance value was measured at 540 nm. Data of one representative experiment out of four independent experiments are shown with mean ± SD $*P<0.05$ and $***P<0.001$; Student's *t*-test.

of the predicted target site completely abolished the effect of miR-31 on reporter gene expression. These data demonstrate that miR-31 directly regulates EMP-1 expression by binding to the predicted target site in its 3′-UTR. In line with this, we found that overexpression of miR-31 in keratinocytes significantly decreased EMP-1 expression, whereas inhibition of miR-31 led to an increased expression of EMP-1, both at mRNA (Figure 4d and Supplementary Figure S6 online) and protein levels (Figure 4e). Next, we analyzed the expression of EMP-1 in human *in vivo* wounds collected at different stages of the healing process. EMP-1 expression level was rapidly increased 3.5-fold ($P=0.001$) 1 day after injury in comparison with the unwounded skin as measured by qRT-PCR; however, its expression returned to basal levels 7 days after injury (Figure 4f). Notably, there was a significant negative correlation ($R=-0.76$, $P=0.0071$) between the expression of EMP-1 and mature miR-31 in the human wound biopsies 1 and 7 days after injury (Figure 4g). Using immunofluorescence staining, we observed that EMP-1 protein was expressed by all layers of epidermal keratinocytes in unwounded human skin, and its signal was stronger in the granular layers (Figure 4h). In line with the qRT-PCR data, the expression of EMP-1 was highly upregulated 1 day after injury and diminished 7 days post wounding (Figure 4h). This reciprocal expression pattern of miR-31 and EMP-1 both *in vitro* and *in vivo* supports that miR-31 targets EMP-1 in keratinocytes and the increased expression of miR-31 may be critical in reducing the EMP-1 level during skin wound healing.

Silencing of EMP-1 promotes keratinocyte proliferation and migration

The function of EMP-1 in keratinocytes is largely unexplored. To determine whether the effects of miR-31 on keratinocyte proliferation and migration could be mediated through EMP-1, we silenced the expression of EMP-1 in keratinocytes by transfection of two different EMP-1-specific small interfering RNAs (siRNAs)—i.e., EMP-1 si-1 and EMP-1 si-2. The silencing efficiency of both siRNAs was confirmed by qRT-PCR (Figure 5a) and western analysis (Figure 5b). Reduced expression of EMP-1 by siRNAs led to a significant increase in

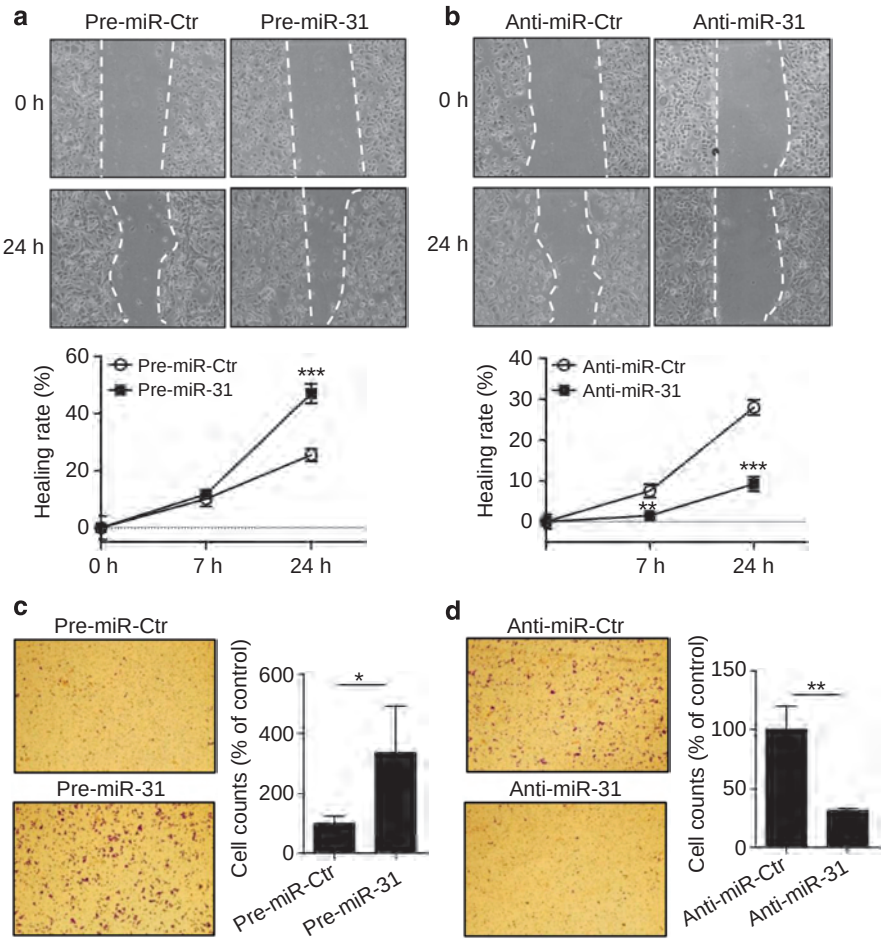

Figure 3. MiR-31 promotes keratinocyte migration. Scratch assays were performed to assess the migration rate of keratinocytes transfected with 20 nM pre-miR-31 (**a**) or anti-miR-31 (**b**) for 48 hours. Photographs were taken at indicated time points after scratch injury. The healing rates were quantified by measuring the area of the injured region. Representative photographs of the transwell migration assay for the keratinocytes transfected with 20 nM pre-miR-31 (**c**) or anti-miR-31 (**d**) were taken under original magnification ×100. The number of keratinocytes passing through the membrane was counted. Data of one representative experiment out of four independent experiments are shown with mean ± SD $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$; Student's *t*-test.

the level of proliferation marker Ki-67 (Figure 5c) and the colony formation capacity of keratinocytes (Figures 5d and e), indicating that EMP-1 inhibits keratinocyte growth. Moreover, the results of scratch assay showed that silencing of EMP-1 increased the migratory capacity of keratinocytes (Figures 5f and g). Taken together, silencing of EMP-1 promotes proliferation and migration of keratinocytes, which phenocopies the effects of miR-31 overexpression. These data indicate that EMP-1 is an important target mediating the biological functions of miR-31 in keratinocytes.

MiR-31 expression is induced by TGF-β2 in keratinocytes

It has been shown that both TGF-β1 and TGF-β2 are crucial cytokines in regulating keratinocyte migration and proliferation during re-epithelialization, and their levels are upregulated in skin wounds (Ramirez *et al.*, 2014). We have previously identified TGF-β1 as an inducer of miR-31 expression in keratinocytes (Xu *et al.*, 2012). Here we evaluated the effect of TGF-β2 on miR-31 expression, showing that it significantly increases the expression of both miR-31 (1.4-fold, $P=0.037$; Figure 6a and Supplementary Figure S7 online) and its primary precursor pri-miR-31 (4.4-fold, $P=0.0001$; Figure 6b) in keratinocytes. Moreover, when we treated keratinocytes with the TGF-β receptor inhibitor SB431542, the inducible effect of TGF-β2 on miR-31 expression was abolished (Figures 6c and d). Accordingly, we found that silencing of TGF-β2 expression in keratinocytes with siRNAs decreased the expression of both miR-31 and pri-miR-31 (Figures 6e and f), indicating that the TGF-β2-mediated-regulation of miR-31 expression also involves autocrine-signaling pathways. In line with this, we show that TGF-β2 significantly inhibited EMP-1 expression in keratinocytes, which may at least partially be mediated by the increased expression of miR-31 (Figure 6g). Interestingly, we found that silencing of EMP-1 expression decreased miR-31 expression in keratinocytes (Supplementary Figure S8 online), suggesting that the high level of EMP-1 during the inflammatory phase may be another trigger factor of miR-31 expression

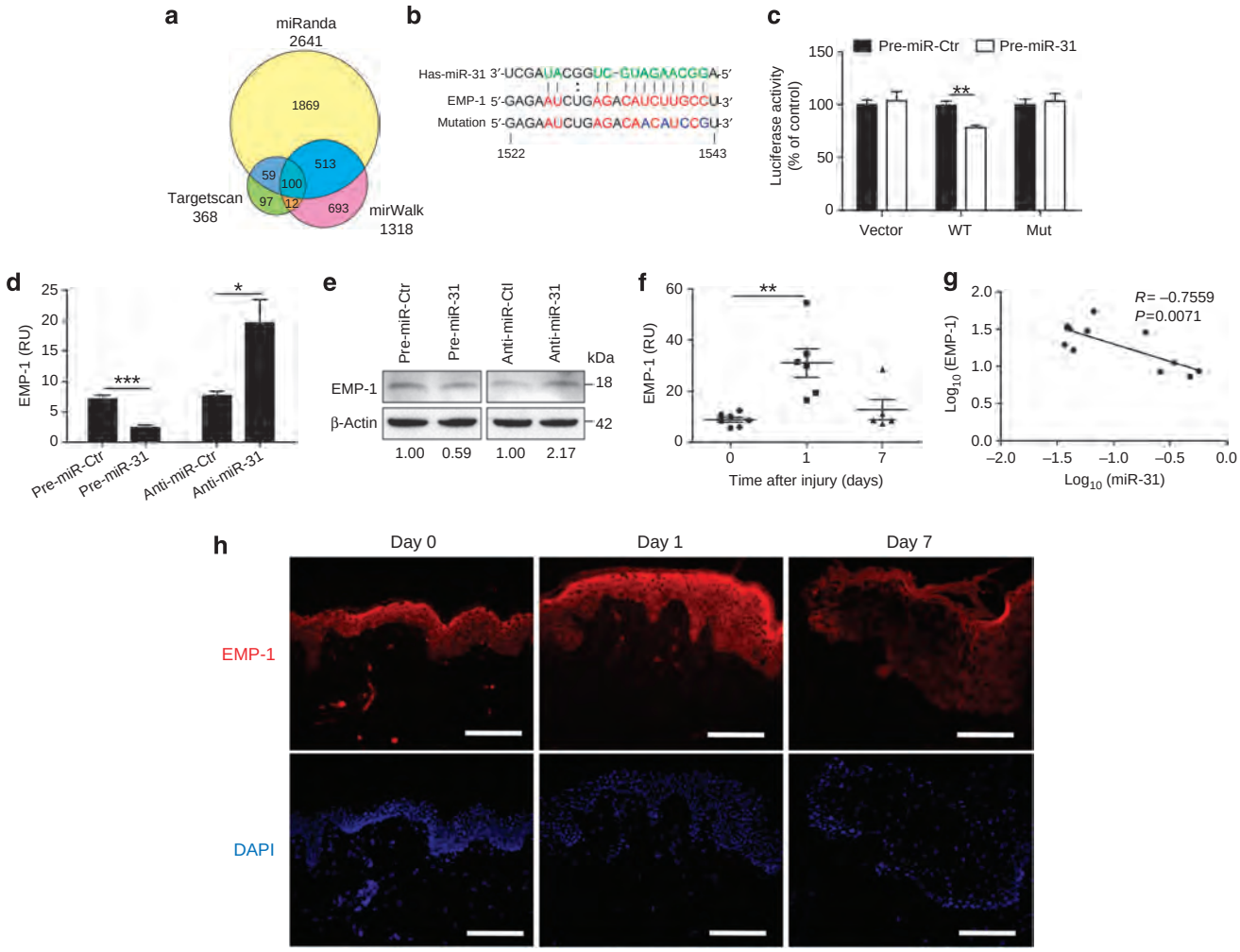

Figure 4. Epithelial membrane protein 1 (EMP-1) is targeted by miR-31 in keratinocytes. (**a**) Venn diagram depicting the number of potential targets of miR-31 predicted by three bioinformatics methods. (**b**) Nucleotide resolution of the predicted miR-31 binding site in 3′UTR of EMP-1 mRNA. (**c**) Keratinocytes were transfected with 200 ng ml^{-1} luciferase reporter plasmids containing wild-type (WT) or mutant (Mut) EMP-1 3′-UTR or empty vector (Vector) together with 20 nM pre-miR-31 or pre-miR-control (Ctr) and luciferase activity was measured 48 hours later. (**d**–**e**) Keratinocytes were transfected with 20 nM pre-miR-31/anti-miR-31 for 48 hours and EMP-1 expression was analyzed by qRT-PCR and western blotting. Data of one representative experiment out of four independent experiments are shown with mean ± SD *$P<0.05$, **$P<0.01$, and ***$P<0.001$; Student's *t*-test. (**f**) EMP-1 expression was analyzed in wound biopsies from healthy donors ($n=7$) before or 1 and 7 days after injury using qRT-PCR. Data are expressed in relative units (RU) compared with 18 s rRNA with mean ± SD **$P<0.01$; the Mann–Whitney *U*-test. (**g**) Correlation of EMP-1 mRNA with miR-31 expression in human wound biopsies 1 and 7 days after injury, Spearman-correlation on log-transformed values. (**h**) Immunofluorescence staining of EMP-1 (red) in human wound sections ($n=7$), which were counterstained using DAPI (blue, nucleus). Scale bar = 100 μm. 3′-UTR, 3′-untranslated region; DAPI, 4′,6-diamidino-2-phenylindole; qRT-PCR, real-time quantitative reverse transcription–PCR; rRNA, ribosomal RNA.

in addition to TGF-β. Collectively, our results suggest that the increased levels of TGF-β in wounds contribute to the upregulation of miR-31 in the keratinocytes.

DISCUSSION

In this study, we characterize the miR-31 expression during human *in vivo* wound healing process. We show that miR-31 is mainly expressed in epidermal keratinocytes, in accordance with our previous observation (Xu *et al.*, 2012). The expression of miR-31 is low in the intact skin; however, upon wounding, its level gradually increases from the inflammatory phase through the proliferative phase. In comparison with the expression pattern of its primary transcript, pri-miR-31, which is only upregulated in the proliferative phase, we conclude that miR-31 may be regulated during wound healing at different levels: the raised amounts of mature miR-31 in the inflammatory phase is most likely due to the more efficient processing of existing pri-miR-31, whereas in the subsequent proliferative phase the further upregulation of miR-31 expression also requires increased transcription of the *miR-31* gene.

The next question was whether this characteristic expression pattern of miR-31 correlates with its biological function during skin wound healing. By using multiple functional assays in human primary keratinocytes with modified miR-31 expression, we demonstrate that miR-31 promotes both the proliferation and migration of keratinocytes, which are crucial

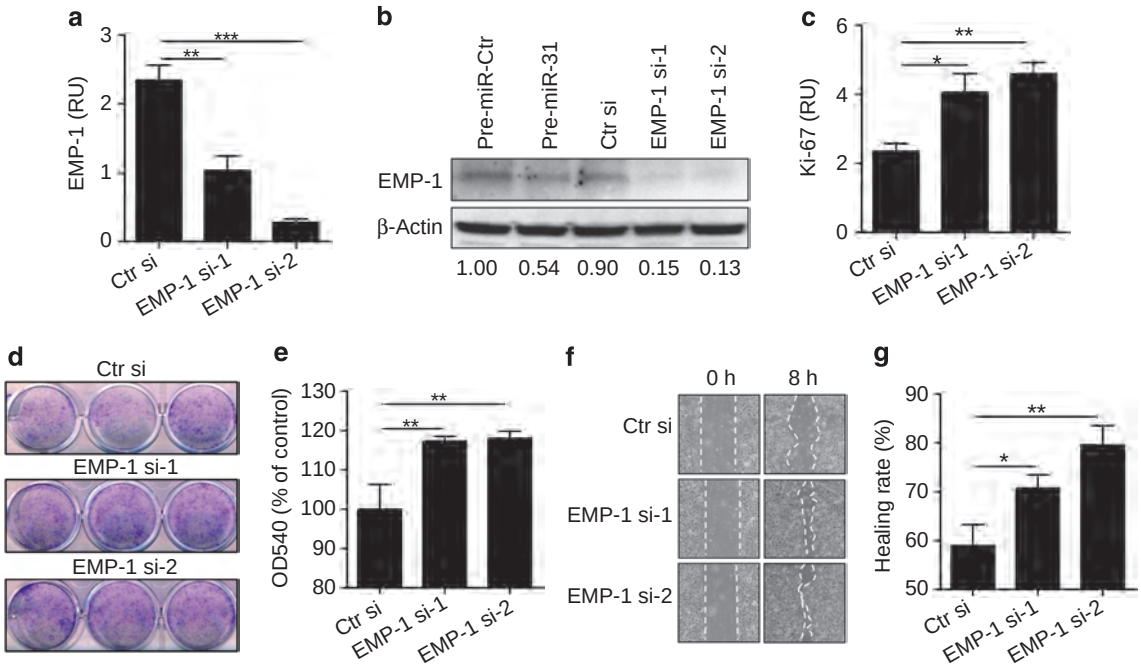

Figure 5. Silencing of epithelial membrane protein 1 (EMP-1) expression increases keratinocyte proliferation and migration. Keratinocytes were transfected with 30 nM EMP-1 small-interfering RNAs (EMP-1 si-1 or EMP-1 si-2) or siRNA-negative control (Ctr si) for 48 hours. The silencing efficiency was analyzed by measuring EMP-1 mRNA with qRT-PCR (**a**) and western blotting (**b**). (**c**) The expression of proliferation marker Ki-67 was analyzed in these transfected cells. Data are expressed in relative units (RU) compared with 18 s rRNA with mean ± SD colonies formed by the transfected keratinocytes were photographed (**d**) and quantified (**e**). Scratch assays were performed to assess the migration rate of keratinocytes transfected with EMP-1 siRNAs. Photographs were taken at indicated time points after scratch injury (**f**), and the healing rates were quantified (**g**). Data of one representative experiment out of four independent experiments are shown with mean ± SD $*P<0.05$, $**P<0.01$, and $***P<0.001$; Student's *t*-test.qRT-PCR, real-time quantitative reverse transcription–PCR; rRNA, ribosomal RNA.

cellular events during the proliferative phase of wound healing and essential for re-epithelialization. Migration of keratinocytes at the wound edge is stimulated by lack of contact inhibition and many factors active during wound healing—e.g., epidermal growth factor, keratinocyte growth factor, and TGF-βs (Reinke and Sorg, 2012). Our study adds miR-31 to a growing number of miRNAs implicated in the regulation of keratinocyte migration—e.g., miR-21 (Ahmed *et al.*, 2011; Yang *et al.*, 2011), miR-203 (Viticchie *et al.*, 2012), miR-483-3p (Bertero *et al.*, 2011), and miR-24 (Amelio *et al.*, 2012). Keratinocytes in the wound margins with mitotic ability, which may include epidermal stem cells, transiently amplifying cells and early differentiated cells (Li *et al.*, 2004), start to proliferate toward the end of inflammatory phase and provide more cells for migration to close the wound (Morasso and Tomic-Canic, 2005). Thus, the high expression of miR-31 in keratinocytes in the proliferative phase may contribute to the re-epithelialization process.

In addition to its effects on keratinocyte growth and motility during wound healing, miR-31 is involved in several other physical processes of the skin. During the hair cycle, miR-31 is upregulated in the anagen phase and inhibition of miR-31 accelerates anagen development (Mardaryev *et al.*, 2010). Also, miR-31 regulates epidermal keratinocyte differentiation by enhancing Notch signaling (Peng *et al.*, 2012). Moreover, the upregulation of miR-31 has been reported in several skin diseases, such as psoriasis, which shares many features with wound healing (Morhenn *et al.*, 2013). MiR-31 is overexpressed in epidermal keratinocytes of psoriasis patients, promoting cytokine and chemokine production by keratinocytes, and contributes to skin inflammation (Xu *et al.*, 2012). Recently, the increased expression of miR-31 was found in cutaneous squamous cell carcinoma, and it functions as an oncomiR by increasing the motility and colony-forming ability of cancer cells (Bruegger *et al.*, 2013; Wang *et al.*, 2014). Taken together, miR-31 is a multifunctional miRNA playing important roles in both physiological and pathological conditions of epidermal keratinocytes.

Identification of the direct target of miR-31 is critical for understanding its mechanism of action in keratinocytes. In this study, we demonstrate that miR-31 targets EMP-1 in keratinocytes, in accordance with a previous report in esophageal squamous cell carcinoma (Zhang *et al.*, 2011). In line with this, we observed a reciprocal expression pattern between miR-31 and EMP-1 in the epidermal keratinocytes at human wound edge during healing process, indicating that miR-31 may control the level of EMP-1 *in vivo*. However, in the inflammatory phase, the expression of miR-31 is only increased slightly, which cannot explain the surge in EMP-1 expression at this time point. Presumably EMP-1 expression may be triggered by other unknown mechanisms. Previously, EMP-1 has mainly been described as a tumor suppressor—e.g., it inhibits tumor cell growth and motility in gastric carcinoma (Sun *et al.*, 2014b), colorectal carcinoma

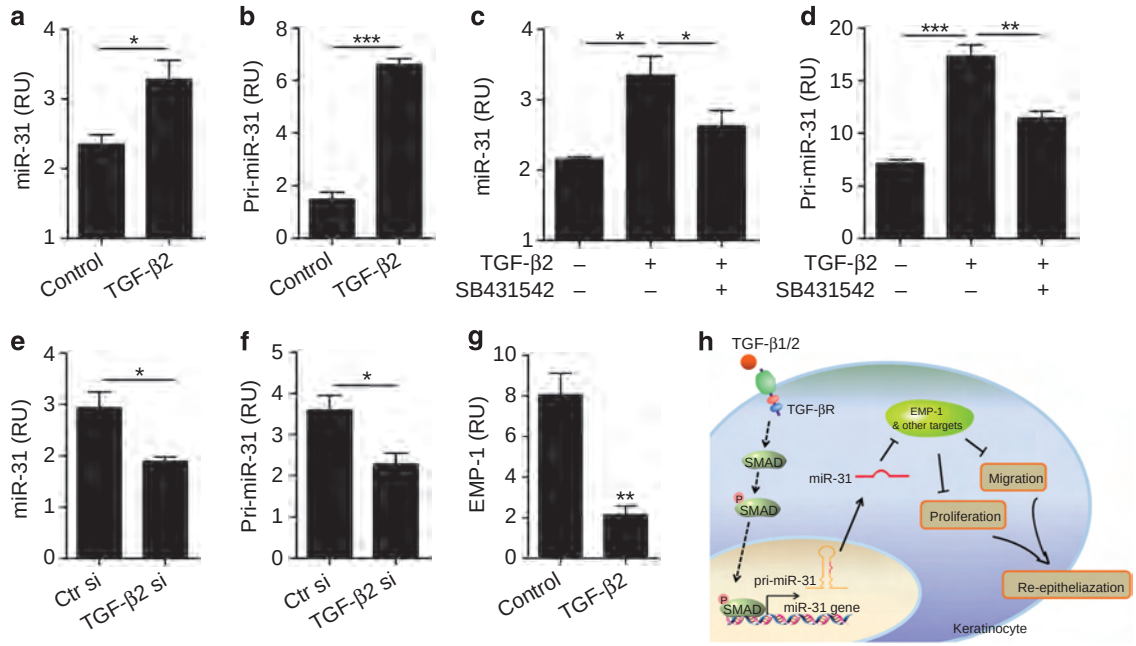

Figure 6. Transforming growth factor-β2 (TGF-β2) induces miR-31 expression in keratinocytes. The expressions of miR-31 (**a**) and of pri-miR-31 (**b**) were analyzed in keratinocytes treated with 10 ng ml^{-1} TGF-β2 for 48h by qRT-PCR. TGF-β receptor inhibitor, SB431542, was applied 15 min before adding TGF-β2. MiR-31 (**c**) and pri-miR-31 expressions (**d**) were analyzed 48 hours later using qRT-PCR. Keratinocytes were transfected with TGF-β2 siRNA for 48 hours and the expressions of miR-31 (**e**) and of pri-miR-31 (**f**) were measured by qRT-PCR. (**g**) EMP-1 expression was analyzed in keratinocytes treated with 10 ng ml^{-1} TGF-β2 by qRT-PCR. miRNA and mRNA PCR data are expressed in relative units (RU) compared with RNU48 RNA and 18S rRNA, respectively. Data of one representative experiment out of four independent experiments are shown with mean ± SD $*P<0.05$, $**P<0.01$, and $***P<0.001$; Student's *t*-test. (**h**) Schematic summary of the regulation and function of miR-31 during skin wound healing. EMP-1, epithelial membrane protein 1; miRNA, microRNA; qRT-PCR, real-time quantitative reverse transcription–PCR; rRNA, ribosomal RNA; siRNA, small interfering RNA.

(Sun *et al.*, 2014c), breast carcinoma (Sun *et al.*, 2014d), nasopharyngeal cancer (Sun *et al.*, 2013), and esophageal squamous cell carcinoma (Zhang *et al.*, 2011). Here we found that EMP-1 also suppressed the growth and migration of normal epidermal keratinocytes. During the transition from the inflammatory to the proliferative phase, the expression of miR-31 is highly upregulated, which results in the reduced expression of its target, EMP1, and thus assuring proper re-epithelialization during skin wound healing. Therefore, in this study, we identified a mechanism mediating the biological functions of miR-31 in skin wound repair—i.e., miR-31 unblocks the EMP-1-dependent repression of keratinocyte proliferation and migration.

Another important question is why miR-31 is upregulated in the wound edge keratinocytes during the healing process. Previously, we performed a systematic screen using different cytokines, growth factors, and factors modulating cell differentiation and identified TGF-β1 as a potent regulator of miR-31 expression in keratinocytes (Xu *et al.*, 2012). Here we expanded the previous findings and show that TGF-β2 can also induce the expression of both mature miR-31 and primary transcripts of miR-31. Binding of TGF-βs to their receptors leads to the activation of downstream Smad proteins, which induce the expression of TGF-β target genes (Ramirez *et al.*, 2014), e.g., miR-31, as shown in our study. It has been known that, upon injury, both TGF-β1 and TGF-β2 are highly upregulated in the skin wounds, promoting keratinocyte migration and re-epithelialization (Ramirez *et al.*, 2014), and these effects may be at least partially mediated through induction of miR-31 expression. Moreover, we found that silencing of EMP-1 expression decreased the level of miR-31 in keratinocytes, suggesting that the high level of EMP-1 during the inflammatory phase may be another trigger factor of miR-31 expression in addition to TGF-β. The EMP-1-induced-miR-31-expression may function as a negative feedback to 'turn-off' the adverse effects of EMP-1 during the later phase of wound healing.

Taken together, our studies propose a model, in which miR-31 is induced by TGF-β1 and TGF-β2 in wound edge keratinocytes. Overexpression of miR-31 increases the proliferation and migration of keratinocytes at least partially by targeting EMP-1 (Figure 6h). These findings suggest that increasing the level of miR-31 in skin wounds may be a strategy to improve healing. Thus, miR-31 may be a promising candidate for further investigations of miRNA-based therapeutics.

MATERIALS AND METHODS

RNA extraction, qRT-PCR, *in situ* hybridization, cell culture methods, cell growth, and motility analysis are described in the Supplementary Materials and Methods online.

Human wound biopsies

Healthy volunteers ($n = 17$) were enrolled at Karolinska University Hospital Dermatology Clinic, Stockholm, Sweden (Supplementary Table S1 online). Full-thickness skin wounds were made with a 3-mm biopsy punch in the abdominal region of the healthy volunteers. On day 1 and day 7 after injury, the wound edge area was excised with a 6-mm biopsy punch and snap frozen (Supplementary Figure S1 online). The clinical material was obtained after written informed consent, and the study was approved by the Stockholm Regional Ethics Committee and conducted according to the Declaration of Helsinki Principles.

Laser capture microdissection

Frozen wound biopsies were embedded in Tissue-Tek (Thermo Scientific, Waltham, MA), and 8-μm tissue sections were cut and stained with hematoxylin. Laser capture microdissection was performed with Leica LMD7000 (Leica, Bernried, Germany). RNA from microdissected tissue was purified using the miRNAeasy Mini Kit (Qiagen, Hilde, Germany).

Plasmids mutagenesis and Luciferase reporter assays

Luciferase reporter plasmids containing 3′-UTR of the EMP-1 gene and empty luciferase vector were obtained from Active Motif (Carlsbad, CA). The mutation was generated at the predicted target site of miR-31 using the QuickChange XL site-directed mutagenesis kit (Stratagene, Lo Jolla, CA) according to the manufacturer's instructions. The sequences of PCR primers for mutation are as follows: forward 5′-gagaaagctaataaagaaaagtacggatgttgtctcagattctcccct ggggt-3′; reverse 5′-accccagggagaatctgagacaacatccgtacttttctttattagctt tctc-3′. The mutation was verified by sequencing (Eurofins Genomics, Ebersberg, Germany). Plasmids (200 ng ml^{-1}) were co-transfected with pre-miR-31 or pre-miR-control (Ctr; 20 nM) into human primary keratinocytes using Fugene HD transfection reagent (Promega, Madison, WI). Forty-eight hours later, Luciferase activity was analyzed using a LightSwitch Luciferase Assay Kit (Active Motif) according to the manufacturer's instructions.

Western blotting

EMP-1 protein expression was detected with mouse anti-human EMP-1 antibody (1:1,000; Abcam, Cambridge, UK) and then visualized using horseradish peroxidise (HRP)-conjugated anti-mouse antibody (1:2,000; Dako Cytomation, Glostrup, Denmark). As loading control, the blots were re-probed using HRP coupled anti-human actin antibody (1:20,000) (Sigma-Aldrich, St Louis, MO).

Immunofluorescence staining

Frozen human wound sections (5 μm) were rinsed with phosphate-buffered saline, followed by incubation with 0.03% H_2O_2 for 10 minute. After blocking with 3% bovine serum albumin, the slides were incubated with rabbit anti-human EMP-1 antibody (1:400, Abcam) at 4 °C overnight. Matched IgG isotype controls were included for each staining. Primary antibodies were detected with Alexa Fluor 594-conjugated goat anti-rabbit IgG (H+L; Life Technologies, Carlsbad, CA) and visualized with a fluorescence microscope.

Statistical analysis

All data are presented as mean ± SD. Mann–Whitney *U*-test or Student's *t*-test was used to determine the significance between two groups. Correlation of the expression of different genes was made using Pearson's correlation test on log-transformed data. *P*-values < 0.05 were considered to be statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Ahmed MI, Mardaryev AN, Lewis CJ *et al.* (2011) MicroRNA-21 is an important downstream component of BMP signalling in epidermal keratinocytes. *J Cell Sci* 124:3399–404

Amelio I, Lena AM, Viticchie G *et al.* (2012) miR-24 triggers epidermal differentiation by controlling actin adhesion and cell migration. *J Cell Biol* 199:347–63

Bertero T, Gastaldi C, Bourget-Ponzio I *et al.* (2011) miR-483-3p controls proliferation in wounded epithelial cells. *FASEB J* 25:3092–105

Bruegger C, Kempf W, Spoerri I *et al.* (2013) MicroRNA expression differs in cutaneous squamous cell carcinomas and healthy skin of immunocompetent individuals. *Exp Dermatol* 22:426–8

Dweep H, Sticht C, Pandey P *et al.* (2011) miRWalk–database: prediction of possible miRNA binding sites by "walking" the genes of three genomes. *J Biomed Inform* 44:839–47

Friedman RC, Farh KK, Burge CB *et al.* (2009) Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* 19:92–105

Gosain A, DiPietro LA (2004) Aging and wound healing. *World J Surg* 28:321–6

Ha M, Kim VN (2014) Regulation of microRNA biogenesis. *Nat Rev Mol Cell Biol* 15:509–24

Haase I, Evans R, Pofahl R *et al.* (2003) Regulation of keratinocyte shape, migration and wound epithelialization by IGF-1- and EGF-dependent signalling pathways. *J Cell Sci* 116:3227–38

He L, Hannon GJ (2004) MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet* 5:522–31

Hydbring P, Badalian-Very G (2013) Clinical applications of microRNAs. *F1000Res* 2:136

Iorio MV, Croce CM (2012) microRNA involvement in human cancer. *Carcinogenesis* 33:1126–33

John B, Enright AJ, Aravin A *et al.* (2004) Human microRNA targets. *PLoS Biol* 2:e363

Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120:15–20

Li A, Pouliot N, Redvers R *et al.* (2004) Extensive tissue-regenerative capacity of neonatal human keratinocyte stem cells and their progeny. *J Clin Invest* 113:390–400

Mardaryev AN, Ahmed MI, Vlahov NV *et al.* (2010) Micro-RNA-31 controls hair cycle-associated changes in gene expression programs of the skin and hair follicle. *FASEB J* 24:3869–81

Morasso MI, Tomic-Canic M (2005) Epidermal stem cells: the cradle of epidermal determination, differentiation and wound healing. *Biol Cell* 97:173–83

Morhenn VB, Nelson TE, Gruol DL (2013) The rate of wound healing is increased in psoriasis. *J Dermatol Sci* 72:87–92

Nana-Sinkam SP, Croce CM (2013) Clinical applications for microRNAs in cancer. *Clin Pharmacol Ther* 93:98–104

O'Connell RM, Rao DS, Baltimore D (2012) microRNA regulation of inflammatory responses. *Annu Rev Immunol* 30:295–312

Peng H, Kaplan N, Hamanaka RB *et al.* (2012) microRNA-31/factor-inhibiting hypoxia-inducible factor 1 nexus regulates keratinocyte differentiation. *Proc Natl Acad Sci USA* 109:14030–4

Ramirez H, Patel SB, Pastar I (2014) The role of TGFbeta signaling in wound epithelialization. *Adv Wound Care* 3:482–91

Reinke JM, Sorg H (2012) Wound repair and regeneration. *Eur Surg Res* 49: 35–43

Santoro MM, Gaudino G (2005) Cellular and molecular facets of keratinocyte reepithelization during wound healing. *Exp Cell Res* 304:274–86

Schneider MR (2012) MicroRNAs as novel players in skin development, homeostasis and disease. *Br J Dermatol* 166:22–8

Sen CK, Gordillo GM, Roy S *et al.* (2009) Human skin wounds: a major and snowballing threat to public health and the economy. *Wound Repair Regen* 17:763–71

Sun BK, Siprashvili Z, Khavari PA (2014a) Advances in skin grafting and treatment of cutaneous wounds. *Science* 346:941–5

Sun G, Zhao G, Lu Y *et al.* (2014b) Association of EMP1 with gastric carcinoma invasion, survival and prognosis. *Int J Oncol* 45:1091–8

Sun GG, Lu YF, Fu ZZ *et al.* (2013) EMP1 inhibits nasopharyngeal cancer cell growth and metastasis through induction apoptosis and angiogenesis. *Tumour Biol* 35:3185–93

Sun GG, Wang YD, Cui DW *et al.* (2014c) Epithelial membrane protein 1 negatively regulates cell growth and metastasis in colorectal carcinoma. *World J Gastroenterol* 20:4001–10

Sun GG, Wang YD, Lu YF *et al.* (2014d) EMP1, a member of a new family of antiproliferative genes in breast carcinoma. *Tumour Biol* 35:3347–54

Viticchie G, Lena AM, Cianfarani F *et al.* (2012) MicroRNA-203 contributes to skin re-epithelialization. *Cell Death Dis* 3:e435

Wang A, Landen NX, Meisgen F *et al.* (2014) MicroRNA-31 is overexpressed in cutaneous squamous cell carcinoma and regulates cell motility and colony formation ability of tumor cells. *PLoS One* 9:e103206

Xu N, Meisgen F, Butler LM *et al.* (2012) MicroRNA-31 is overexpressed in psoriasis and modulates inflammatory cytokine and chemokine production in keratinocytes via targeting serine/threonine kinase 40. *J Immunol* 190: 678–88

Yang X, Wang J, Guo SL *et al.* (2011) miR-21 promotes keratinocyte migration and re-epithelialization during wound healing. *Int J Biol Sci* 7:685–90

Zhang T, Wang Q, Zhao D *et al.* (2011) The oncogenetic role of microRNA-31 as a potential biomarker in oesophageal squamous cell carcinoma. *Clin Sci (Lond)* 121:437–47